Australasian Journal of Dermatology

Efficacy and mechanism of action of topical ingenol mebutate 0. 05% gel in basal cell carcinoma.

Journal:	*Australasian Journal of Dermatology*
Manuscript ID	Draft
Manuscript Type:	Original Research
Keywords:	Basal cell carcinoma (BCC), CD4+, CD68+, immune response, ingenol mebutate gel, natural killer cells

INTRODUCTION

Basal cell carcinoma (BCC) is the most common malignancy in the Caucasian population. It accounts for around 80% of all non-melanoma skin cancers (NMSC). It is typically slow growing and rarely metastatic but its location, tendency to relapse, multiplicity and possibility to invade and destroy local tissues[1] delimit disease morbidity. Surgical approaches are the standard strategy for well-defined BCC. Mohs micrographic surgery is generally applied to high-risk tumors or those tumors placed in cosmetically sensitive areas whereas cryotherapy or electrodessication and curettage (EDC) are acceptable treatment options for small size superficial BCC (sBCCs) in the trunk and limbs. However, for patients who are poor candidates for surgery, or have low-risk BCC, as is the case of sBCC, non-surgical methods such as topical treatment or photodynamic therapy may be alternative treatment options which may also reduce the risk of scarring compared to the surgical approach.[2, 3]

Among some already known topical treatments such as 5-fluoracil cream and imiquimod gel, topical ingenol mebutate gel (PEP005), a diterpene ester extracted from the plant *Euphorbia Peplus*, is only approved for actinic keratosis treatment. In actinic keratosis, IMG 0.05% is applied once daily for 2 consecutive days in affected areas of 25 cm^2 of the trunk and limbs. IMG has demonstrated therapeutic effects on various cutaneous neoplasms including warts, corns, and cancerous lesions[4]. Although IMG is not considered the first option for the treatment of BCC; there is some evidence supporting its efficacy and safety[4-11]. In a phase II randomized study, 60 patients with sBCC were placed on varying dosing regimens and concentrations of either IMG or the gel vehicle. Significant histologic clearance at 85 days post-treatment was observed in 63% of patients when 0.05% IMG was applied for two consecutive days ($p<0.031$)[5].

IMG induces mitochondria swelling of dysplastic keratinocytes and cell death by primary necrosis. Topical application generates neutrophilic infiltration due to protein kinase C activation, causing effective wound healing. Its dual mechanism of action is characterized by a rapid necrosis lesion beginning 1 to 2 hours after application (causing an increase of intracellular calcium, mitochondrial swelling, and loss of cells membrane integrity) followed by tumor cell apoptosis via neutrophil-mediated cellular cytotoxicity occurring within days. At this time, there is also an increase of TNF-α and IL-8, which recruits and subsequently activates neutrophils towards the inflammatory infiltrate[12]. However, the exact mechanism of action of IMG has not been fully elucidated.

Here, we describe our experience using topical treatment IMG, 0.05% under occlusion in a group of patients with BCC in low-risk locations (trunk and limbs). Our aim was to assess the efficacy and safety of IMG in this indication but also to characterize the inflammatory cell infiltration at different timepoints to better understand IMG's mechanism of action. To our knowledge, this is the first study that, beyond clinical

evaluation, analyses the immunoinflammatory infiltrate post IMG treatment in BCC tumors.

MATERIALS AND METHODS

Between May and September 2015, a prospective, parallel, interventional, and randomized clinical study on the efficacy of IMG in BCC was conducted. Twenty one patients were chosen for the screening visit of which five of them were excluded due to incorrect treatment compliance: a total of 16 BCC patients were included. Inclusion criteria was: Caucasian adults above 18 years of age with a histologically confirmed primary BCC (superficial, nodular or infiltrating histological subtypes defined in accordance with published criteria[2]), located in low risk zones (trunk and limbs) with an extension up to 1 cm^2. Exclusion criteria was: pregnant and breast feeding women, immunocompromised patients, genetic predisposition to BCC or recurrent BCCs. The study was approved by the Independent Ethics Committee at the Hospital Universitari Arnau de Vilanova de Lleida, Spain.

Informed consents and samples from patients were obtained with support from IRB Lleida Biobank (B.0000682) and PLATAFORMA BIOBANCOS (PT13/0010/0014). Biobank is an authorized institution of the Health Department of Catalonia as from the 29th of April, 2013 and registered in the National Register of Biobanks of the Carlos III Health Institute (Spain). The Biobank guarantees the traceability and quality of the samples, plus the consent process undertaken in accordance with the protocols approved by the Local Ethical Committee, following the basic principles (respect for the individual), operational risk-management (risk-benefit) and guidelines (good clinical practice) of the Declaration of Helsinki (World Medical Association, 1964).

IMG 0.05% was applied on the lesion site and on a 1 cm perimeter surrounding the lesion site, once daily for two consecutive days. Considering BCC as more locally invasive than actinic keratosis, we applied the gel under occlusion with aluminum disks. Lesions were observed in the following days after treatment according to randomization groups: biopsies were taken between the third and the tenth day after treatment initiation in the first group as an ‘early immune response model’, coinciding with the maximum clinical inflammation (10 patients, Group 1) and the second group was biopsied at day 30 after treatment initiation as a ‘late immune response model’ (6 patients, Group 2); (Fig. 1).

Control samples consisted of five biopsies obtained from both groups before starting IMG application (untreated samples). Treatment efficacy was assessed in terms of clinical and histological complete response. We used the local skin reaction (LSR) grading scale to describe side effects related to therapy[11].This scale is based on a 0–4 numerical

index (being 4 the highest grade of severity) related to 6 specific clinical parameters (erythema, scaling, crusting, swelling, and vesiculation/pustulation, erosion/ulceration) accompanied by a characteristic photographic image for each rating. Total LSR (Local Skin Reaction) score ranges from 0 to 24 points[13]. The LSR test was evaluated one week after treatment initiation for every patient. Pictures were also taken in every patient at the screening visit, before treatment initiation, and during the follow up visits depending on each arm. Patients were randomized into group 1 and group 2 according to whether the inclusion visit was an even or odd day. An additional follow up after 3 months, 6 months, 1 year, and 2 years after treatment was conducted in every case. Patients with no tumor clearance in the follow-up biopsies discontinued the study and any remaining tumor was treated by surgery short time later.

Immunohistochemical study

Histopathological changes including necrosis and type and degree of the inflammatory infiltrate were analyzed by a semiquantitative measurements.

All post-treatment biopsies and the 5 screening biopsies (control) were analyzed by immunohistochemistry with different antibodies: antiCD3, -CD4, -CD8, -CD20, -CD56, -CD68, -Bcl-2, -CASP3, -FoxP3, -GrzB and -TIA1. See Table 1.

Tissue blocks were sectioned at 3 µm-thickness, dried for 1 hour at 65ºC before pre-treatment procedure of deparaffinization, rehydration and epitope retrieval in the pre-Treatment Module, PT-LINK (DAKO) (at 95ºC for 20 min in 50 x Tris/EDTA buffer and pH 9). Before staining, endogenous peroxidase activity was blocked with peroxidase blocking solution (Dako, Glostrup, Denmark). After incubation with primary antibodies, the reaction was visualized with the EnVisionTM FLEX Detection Kit (DAKO, Glostrup, Denmark) using diaminobenzidine chromogen as a substrate according to manufacturer's instructions. Sections were counterstained with *hematoxylin. P*rimary antibodies used in this study are listed in Table 1.

All tissue samples were histologically reviewed by 2 blinded members of the team. For the staining scoring, an automated imaging system, the ACIS® III Instrument (DAKO, Denmark, Glostrup), was used. The mean percentage of positive cells was obtained after evaluating regions of interest coincident with areas of higher infiltration.

Immunohistochemical post-treatment results were normalized by a control group and were analyzed by ANOVA statistical test.

RESULTS

Efficacy and safety of IMG 0.05% under occlusion in BCC

A total of sixteen patients were included in the study. Clinical and tumor baseline variables are shown in Table 2. The majority of patients were women (62.5%) with a

mean age of 66 and more than half of the lesions were located in the trunk (56.25%). There were ten superficial BCCs, five nodular BCCs and one infiltrative BCC.

After treatment, complete tumor clearance was observed in 8/10 (80%) sBCC, 2/5 (40%) nodular BCCs and 0/1 (0%) infiltrative BCC. Overall, 10 tumors were cleared which did not show any evidence of recurrence after a 2-year follow-up.

Regarding adverse events, 10/16 (62.5%) patients experienced a significant local inflammatory reaction with marked tumor erosion and the LSR grading scale was over 20. Four out of sixteen (25%) patients had medium LSR score (values between 12-18) and 2/16 (12.5%) patients had low LSR score (LSR score < 8). Interestingly, patients with medium or low values in LSR score did not show clinical nor histological clearance.

The first day of treatment, some patients experienced severe pain and flaking/blistering/erythema extending beyond the application site (Fig. 2). From the third day, pain was gradually relieved (with all symptoms disappearing after 1 week) and an erosive patch developed after blister rupture (Fig. 3).

At the end of treatment (days 3 to 5), erythema was present in 100% of BCCs and erosions and bulla were present in 10/16 of the cases. LSRs lasted about 2 weeks and we observed almost complete resolution after 5 weeks from treatment initiation. No systemic features as headache or malaise were reported.

Overall, this study showed that 10/16 (62.5%) patients were in complete remission after 2 years of follow-up. LSRs started the first day of treatment but resolved almost completely after 5 weeks.

Histopathology

An important epithelial and superficial dermal necrosis was observed in biopsies taken from Group 1 accompanied by an important inflammatory cell infiltration mainly composed of polymorphonuclear (PMN) cells (Fig. 4) and some mononuclear cells. Necrosis degree and inflammatory infiltrate in group 2, were lower than in group 1. In biopsies taken at day 30 after treatment initiation (Group 2) necrosis degree and inflammatory infiltrate were lower than in Group 1, and necrosis was replaced by fibrosis although a mild-moderate mononuclear cell infiltration was still evident in most of the samples (Fig. 4). Regarding the adaptive immune response, a similar pattern was observed. A high increase of CD3+ cells was observed at early timepoints ($p=0.013$ compared to control), mainly composed by cytotoxic CD8+ and CD4+ T cells ($p=0.16$ and $p=0.016$ respectively compared to control). T cell recruitment also decreased over time, but while CD4+ T cells returned to control levels at day 30 after treatment initiation (group 2), residual presence of CD8+ T cells was observed although not statistically significant. These results suggest that some cytotoxic activity was present at the BCC zone at late timepoints considering the presence of CD8+ T cells and NK (CD56+) within the infiltrate. Moreover, remaining expression of TIA-1 (cytotoxic marker) was also observed.

Reduction of T-cell recruitment in the late immune response coincided with a high presence of regulatory T cells (Tregs); FoxP3+ in situ cells. This subpopulation suppresses effector T cells activity (CD4+ and CD8+). There was also some B-cell recruitment (CD20) possibly induced later in the immune response cascade compared with effector T-cell recruitment and remaining for longer time. Although this tendency, presence of both cell subtypes is not statistically significant (data not shown; Fig. 6).

Regarding apoptotic markers, we observe a high expression of active caspase 3 and granzyme B at early timepoints ($p=0.13$ and $p=0.06$ respectively compared to control) whereas the levels of the transcription factor Bcl-2an antiapoptotic marker, were lower in group 2 than in group 1, reaching lower levels than in control samples. This apoptosis might be induced to stop the immune response from all recruited inflammatory cells once the tumor is cleared. (Fig. 5, 6).

DISCUSSION

The incidence of non-melanoma skin cancers is undergoing a drastic global increase. The continuous search for non-invasive treatments has encouraged the development of new therapeutic agents. An understanding of the history, mechanism of action, and recent trial evidence for emerging therapies may help physicians in counseling patients on available treatment options and to select the appropriate therapy [7,8].

In this study we demonstrated the efficacy of IMG 0.05% in BCC under occlusion, especially for the superficial subtype, where 80% of the cases achieved complete remission and maintained this response after 2 years of follow-up.

Published clinical trials where sBCC were treated with IMG show similar or lower response rates: in a phase II randomized study, 60 patients with sBCC were placed on varying dosing regimens and concentrations of either IMG or the gel vehicle. Significant histologic clearance at 85 days post-treatment was observed in 63% of patients when 0.05% IMG was applied for two consecutive days ($p<0.031$)[5]. In a phase I/II clinical study, 82% of patients who failed or refused conventional treatment for sBCC achieved a complete clinical response one month after *Euphorbia peplus* treatment and 57% continued after a mean follow-up of 15 months[6].

Safety profile in those trials was favorable with mild to moderate adverse events including erythema, flaking, scaling, erosion, and ulceration. LSRs appeared to be dose-dependent and developed rapidly after application (within the first day), peaked in

severity shortly after the end of the treatment (one week) and returned to near baseline levels within two weeks[5].

There are also some other case reports[7] and short patient series with BCC, treated with different IMG posology (0.015 or 0.05%), time length and number of cycles with complete clearance outcomes [5-10].

The main difference between our study and those reported in the literature is the use of IMG under occlusion; it may be the reason why we obtained higher rates of complete response in sBCC. High efficacy of IMG 0.05% under occlusion has already been shown in a short series (n=7) where patients with sBCC in the trunk achieved complete response within 2 to 4 weeks[10].

Apart from sBCC cases, our study included other BCC subtypes (superficial, nodular or infiltrating histological subtypes). The results indicate that 2/5 (40%) nodular BCC resolved whereas none (0/1; 0%) of the infiltrative BCC had a successful response. We may conclude that IMG under occlusion was more efficacious in treating sBCC rather than other subtypes despite the limitation of having collected very low number of lesions. To our knowledge there is not much evidence of nodular or infiltrative BCCs treated with IMG reported in the literature. More studies should be done in order to reach stronger conclusions about the use of IMG in those BCC subtypes. Overall, our study showed that 10/16 (62.5%) of patients obtained complete remission after treatment.

We would like to highlight that 2/5 patients that were withdrawn from the study due to incorrect treatment compliance, had tumor residues confirmed by biopsy. However, some weeks later, when those skin lesions were about to be removed by surgery, no tumor was detected at that time. In some cases, the immune system may take longer to completely destroy the tumor since patients that were considered as non-complete responders actually were complete responders. We hypothesize that this finding may correlate with our observation that some cytotoxic cells remained at the tumor site even 30 days after treatment initiation, meaning that some cytotoxic activity may be present at the late immune response timepoint, even after LSRs resolution.

Regarding safety, we observed remarkable adverse events in 10/16 (62.5%) of the patients. Erythema and blister formation started within the first day of treatment and an extensive necrosis was present at day 3 after treatment initiation concurring with a high inflammatory response. IMG produces a non-specific epidermal and superficial dermal necrosis at the very beginning leading to a release of the cytosolic components within the tumor site, acting as antigens triggering the activation of both the innate and the adaptive immune responses. This papillar dermal necrosis produced by occlusive treatment may be responsible for scarring in some patients and might also be the reason why we observed longer duration of LSRs in our patients compared to other studies

found in the literature. It is also important to notice that 6 patients with medium or low LSR score did not show tumor regression, suggesting that an incomplete immune response is unable to induce tumor clearance.

Regarding cell recruitment at tumor site, in terms of innate response we observed a high neutrophilic infiltration (PMN) reinforced by macrophages (p=0.03) and NK cells (even if not statistically significant).

In terms of adaptive immune response, we observed an important recruitment of T-cells (lymphocytes): CD4+ (p=0.016) and CD8+ (non statistically significant) at early timepoints. Interestingly, CD4+ T cells return to control levels whereas the recruitment of NK cells, CD8+ T cells, and the presence of TIA-1 show that some cytotoxic activity may remain within the tumor site at late immune response timepoints.

There is also a high expression of active caspase 3 and granzyme B at early timepoints (10 days after treatment initiation; data not shown), at the same time when we observed an increase of Tregs cells; a T-cell subpopulation able to downregulate and suppress T effector cells by several mechanisms, including apoptosis. The real mechanism of action of Tregs cells has not been fully elucidated[14], but Tregs cells may induce the apoptotic activity to decrease the recruitment and activity of T effector cells (CD4+ and CD8+) as we observe.

Similar results were obtained in a phase I randomized trial in which 26 patients with actinic keratosis were treated with IMG 0.05%. One day after treatment initiation, inflammatory cell infiltration was dominated by CD4+ and CD8+ T cells as well as neutrophils and macrophages within both dermis and epidermis. Fewer changes were observed for CD20+ B-cells which might be produced later, as we observed in our study. Apoptosis (caspase 3) was also found at early timepoints after treatment.[15]

Overall, we found that ingenol mebutate gel was an effective drug to treat basal cell carcinoma, especially the superficial subtype. Necrosis reaction accompanied by both innate and adaptive immune cells recruitment mainly occurring at early response timepoints after treatment initiation may be responsible for the observed efficacy rates. Cytotoxic markers observed at late response timepoints may ensure complete response by destroying residual tumor cells. Further research about the ingenol mebutate mechanism of action needs to be done.

ACKNOWLEDGEMENTS

This was an investigator initiated study funded by LEO Pharma. The design of the study and analysis and interpretation of study results are exclusive responsibility of investigators. Work supported by IRBLleida Biobank (B.0000682) and PLATAFORMA BIOBANCOS (PT13/0010/0014). We would like to thank Lidia Rodriguez, Scientific Affairs LEO Pharma SA for support and medical writing.

REFERENCES

1. Trakatelli M, Morton C, Nagore E *et al*. BCC subcommittee of the Guidelines Committee of the European Dermatology Forum. Update of the European guidelines for basal cell carcinoma management. *Eur J Dermatol* 2014; 24: 312-29.

2. Clark CM, Furniss M, Mackay-Wiggan JM. Basal cell carcinoma: an evidence-based treatment update. *Am J Clin Dermatol* 2014; 15: 197-216.

3. Jansen MHE, Mosterd K, Arits AHMM, Roozeboom MH, Sommer A, Essers BAB. Five-year results of a randomized controlled trial comparing effectiveness of photodynamic therapy, topical imiquimod and topical 5-fluorouracil in patients with superficial basal cell carcinoma. *J Invest Dermatol* 2017; (Epub ahead of print)

4. Cantisani C, Paolino G, Cantoresi F, Faina V, Richetta AG, Calvieri S. Superficial basal cell carcinoma successfully treated with ingenol mebutate gel 0,05%. *Dermatol Ther* 2014; 27: 352-4.

5. Siller G, Rosen R, Freeman M, Welburn P, Katsamas J, Ogbourne SM. PEP005 (ingenol mebutate) gel for the topical treatment of superficial basal cell carcinoma: results of a randomized phase IIa trial. *Australas J Dermatol* 2010; 51: 99-105.

6. Ramsay JR, Sunrbier A, Aylward JH *et al*. The sap from Euphorbia peplus is effective against human nonmelanoma skin cancers. *Br J Dermatol* 2011; 164: 633-6.

7. Jung YS, Lee JH, Bae JM, Kim GM. Superficial Basal Cell Carcinoma Treated with Two Cycles of Ingenol Mebutate Gel 0,015. *Ann Dermatol* 2016; 258: 796-7.

8. Diluvio L, Bavetta M, Di Prete M, Orlandi A, Bianchi L, Campione E. Dermoscopic monitoring of efficacy of ingenol mebutate in the treatment of pigmented and non-pigmented basal cell carcinomas. *Dermatol Ther* 2017; 2017 Jan; 30(1). doi: 10.1111/dth.12438. Epub 2016 Nov 12.

9. Izzi S, Sorgi P, Piemonte P, Carbone A, Frascione P. Successfully treated superficial basal cell carcinomas with ingenol mebutate 0, 05% gel: Report of twenty cases. *Dermatol Ther* 2015; 29: 470-2.

10. Bettencourt MS. Treatment of superficial basal cell carcinoma with ingenol mebutate gel, 0, 05%. *Clin Cosmet Investig Dermatol* 2016; 9: 205-9.

11. Del Rosso JQ. Ingenol Mebutate Topical Gel A Status Report On Clinical Use Beyond Actinic Keratosis. *J Clin Aesthet Dermatol* 2016; 9:S3-S11.

12. Hui A, Markowitz O. Ingenol mebutate for the treatment of actinic keratosis. *Curr Derm Rep* 2013; 2: 172–6.

13. Rosen R, Marmur E, Anderson L, Welburn P, Katsamas J. A new, objective, quantitative scale for measuring local skin responses following topical actinic keratosis therapy with ingenol mebutate. *Dermatol Ther (Heidelb)* 2014; 4: 207-19.

14. Yolcu ES, Ash S, Kaminitz A, Sagiv Y, Askenasy N, Yarkoni S. Apoptosis as a mechanism of T-regulatory cell homeostasis and suppression. *Immunol Cell Biol* 2008; 86: 650-8.

15. Emmert S, Haenssle HA, Zibert JR et al. Tumor-Preferential Induction of Immune Responses and Epidermal Cell Death in Actinic Keratoses by Ingenol Mebutate. *PLoS One* 2016; 11:e0160096.

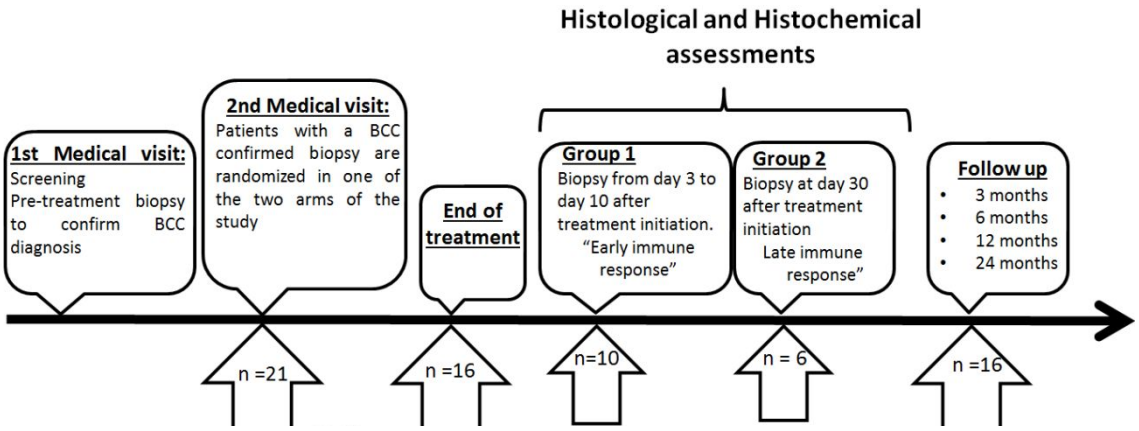


Figure 1. Study design (timing and assessment visits).

Antigen	Clone	Catalog number	Dilution	Source	Cell marker
CD3	Polyclonal	IR503	Ready to use	DAKO, Glostrup, Denmark	T lymphocytes
CD4	4B12	IR649	Ready to use	DAKO, Glostrup, Denmark	Helper lymphocytes
CD8	C8/144B	IR623	Ready to use	DAKO, Glostrup, Denmark	Cytotoxic lymphocytes
CD20	L26	IR604	Ready to use	DAKO, Glostrup, Denmark	B lymphocytes
CD56	123C3	IR628	Ready to use	DAKO, Glostrup, Denmark	Natural killers lymphocytes
CD68	PG-M1	IR613	Ready to use	DAKO, Glostrup, Denmark	Macrophages
Bcl-2	124	IR614	Ready to use	DAKO, Glostrup, Denmark	Antiapoptotic protooncogen
Cleaved caspase 3 (CASP3)	Polyclonal	9661	1:100	Cell signaling, Massachusetts, USA	Apoptotic marker
FoxP3	D2W8E	98377	1:100	Cell signaling, Massachusetts, USA	Regulatory lymphocytes
Granzyme B (GrzB)	GrB-7	M7235	1:50	DAKO, Glostrup, Denmark	Cytotoxicity marker
TIA1	TIA-1	Ab2712	1:100	Abcam, Cambridge, UK	Natural killers and cytotoxic lymphocytes

Table 1. Primary antibodies used in the study to evaluate inflammatory cell infiltration in groups 1 and 2 after treatment with IMG.

Patients	n (%)
Men	6 (37.5%)
Women	10 (62.5%)
Mean Age	66
BCC subtype	**n (%)**
Superficial	10 (62.5%)
Nodular	5 (31.25%)
Infiltrative	1 (6.25%)
Localization	**n (%)**
Trunk	9 (56.25%)
Abdomen	1 (6.25%)
Neck	2 (12.5%)
Upper extremities	0
Lower extremities	4 (25%)

Table 2. Clinical and pathological data from the 16 patients with BCC included in the study.

Figure 2. Local skin reaction after IMG treatment**.** (a) Nodular BCC in lower limbs. (b) Clinical reaction after 3 days from treatment initiation. Note the extending erythema with an important blister in the middle which produced severe pain to the patient. (c) Clinical appearence 30 days from treatment initiation (group 2). (d) Clinical appearence after 6 months of follow up; no evidence of recurrence was detected but a residual hiperpigmentation and a scar were observed.

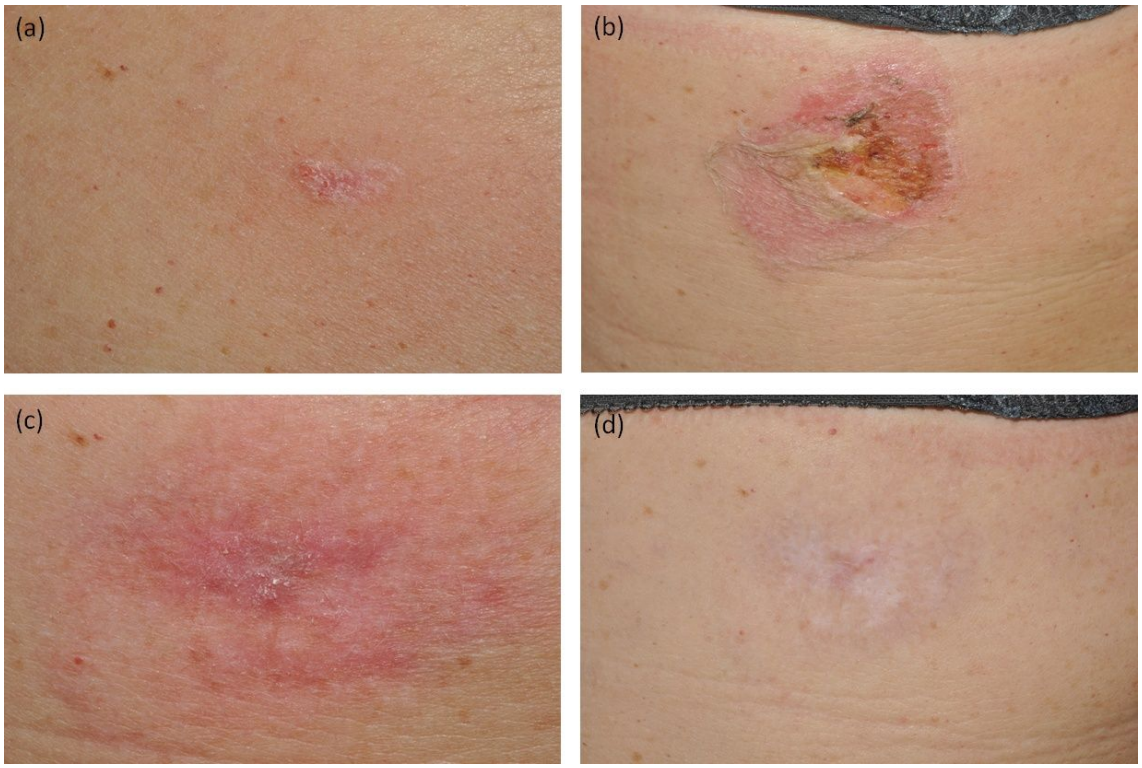


Figure 3. Follow up of a patient treated with IMG. (a) Superficial BCC in the back at baseline. (b) An erosive patch after blister rupture was observed 10 days after treatment. (c) Residual erythema after 2 months of treatment initiation. (d) Residual scar after 6 months of follow up.

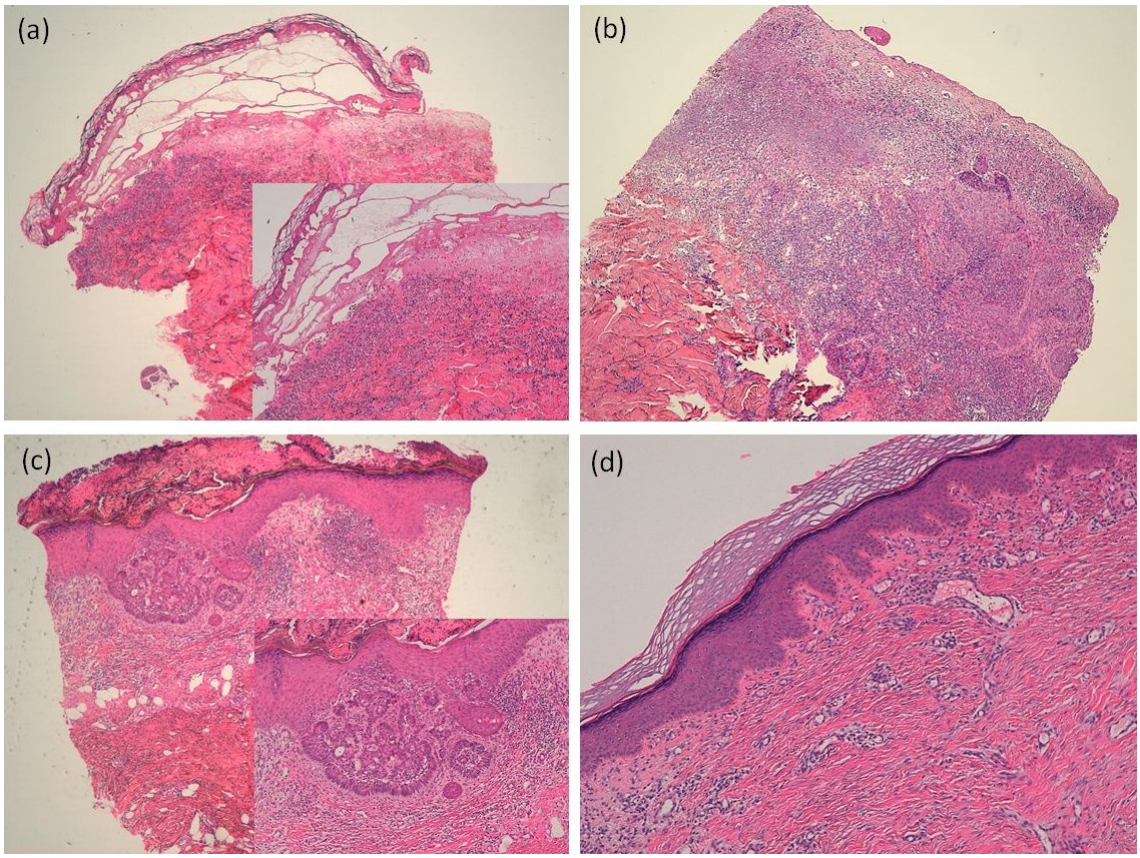


Figure 4. Representative samples showing different degrees of necrosis according to the timepoint when biopsies were collected. (a,b) Relevant epidermal and dermal necrosis appearing in certain areas reaching the middle dermis after 3 days from treatment initiation with IMG were observed. Image (a) emphasizes a huge sub-epidermal blister while image (b) emphasizes the intense neutrophilic infiltrate accompanied by an *important inflammatory cell infiltration mainly composed by polymorphonuclear cells*. (c) Reduction of necrosis and the intensity of the immune cell infiltration were observed 10 days after treatment initiation with IMG. Some tumor areas were still present. (d) Day 30 post-treatment biopsy. A residual immune cell infiltration and fibrosis tissue were observed. Original magnification: (a,b,c) Hematoxylin and eosin (4X); (d) Hematoxylin and eosin (10X).

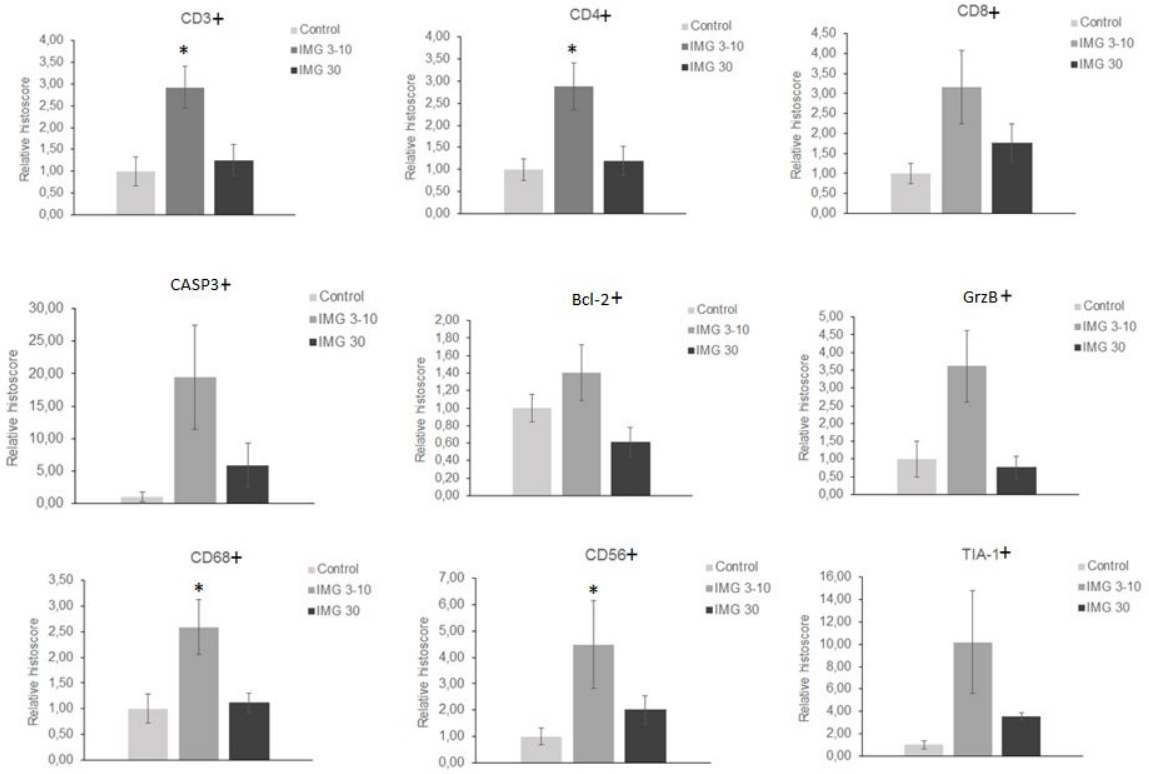


Figure 5. Bar chart with the results of all analyzed markers. ANOVA statistical test, * =P<0.05.

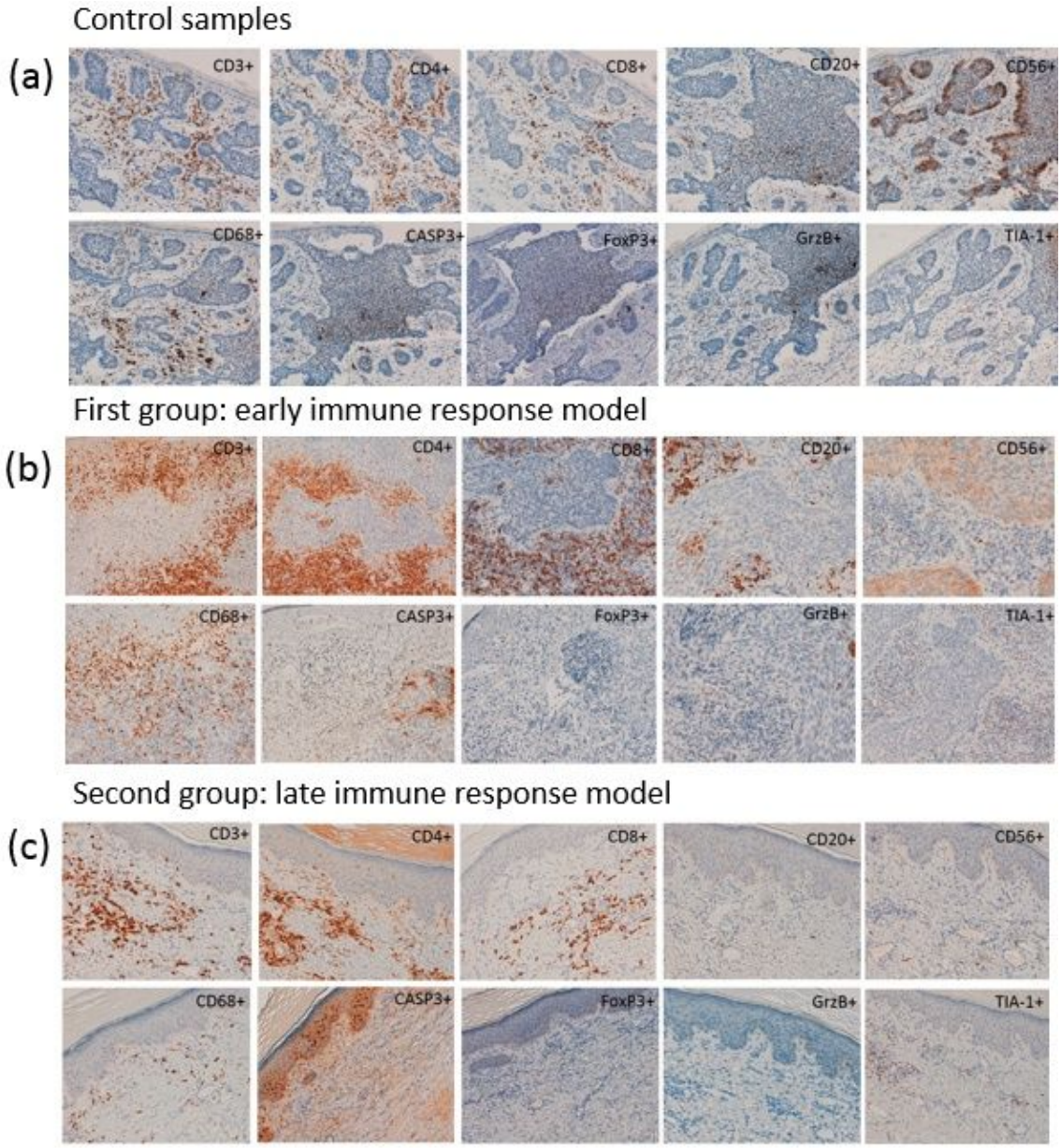
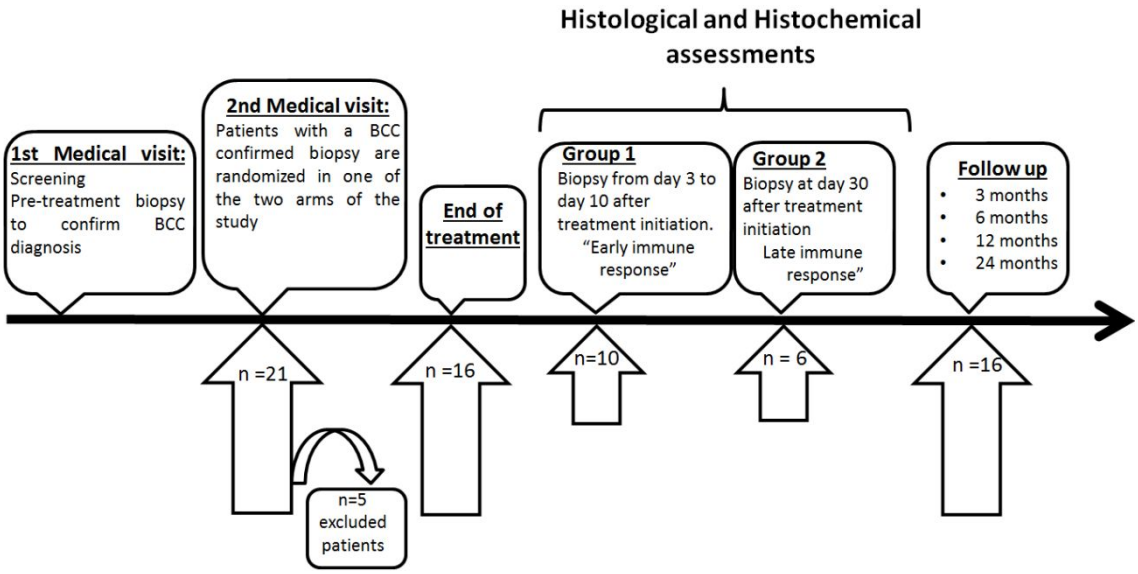


Figure 6. Representative samples showing immunohistochemistry results in the control samples and in the different groups. (a) Control samples. (b) Group 1(early immune response). (c) Group 2 (late immune response).



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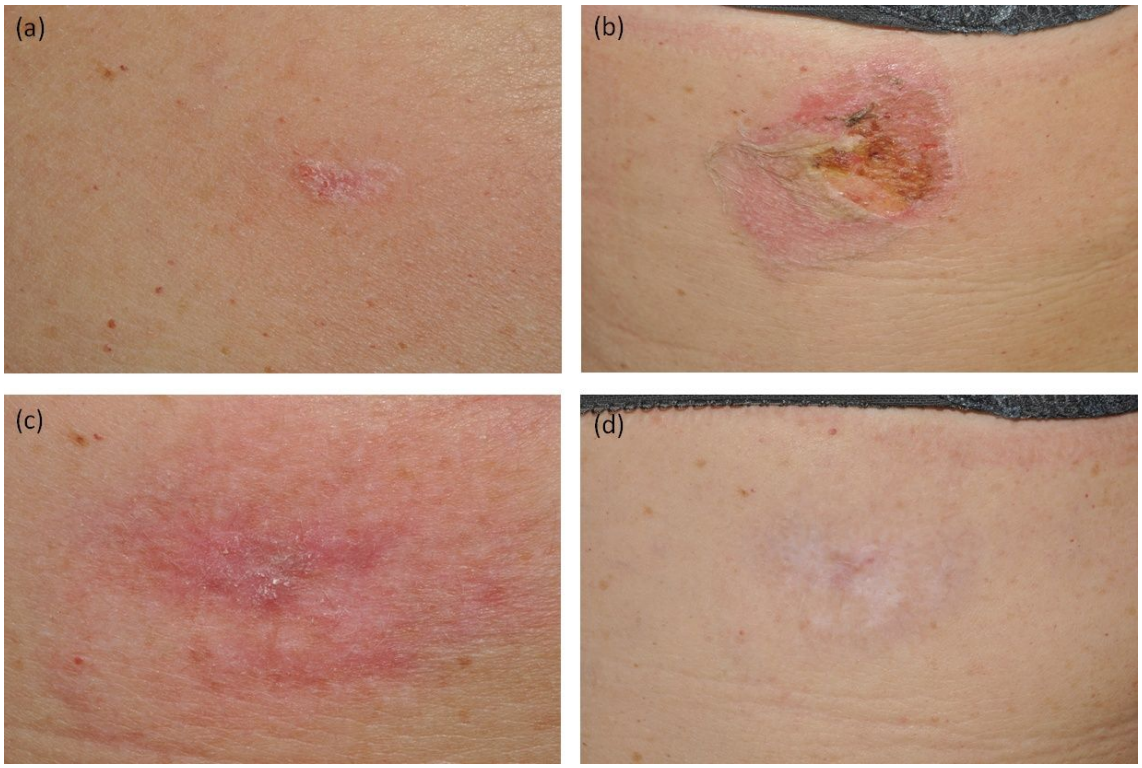

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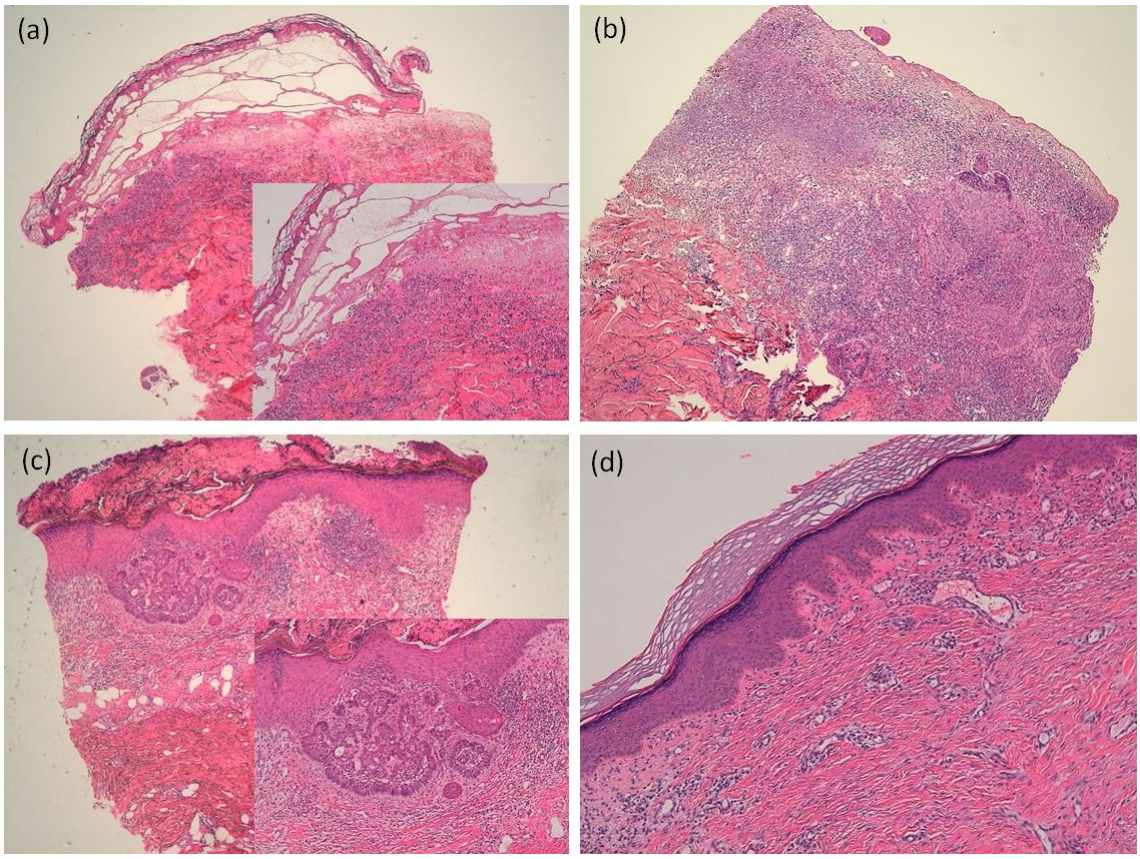

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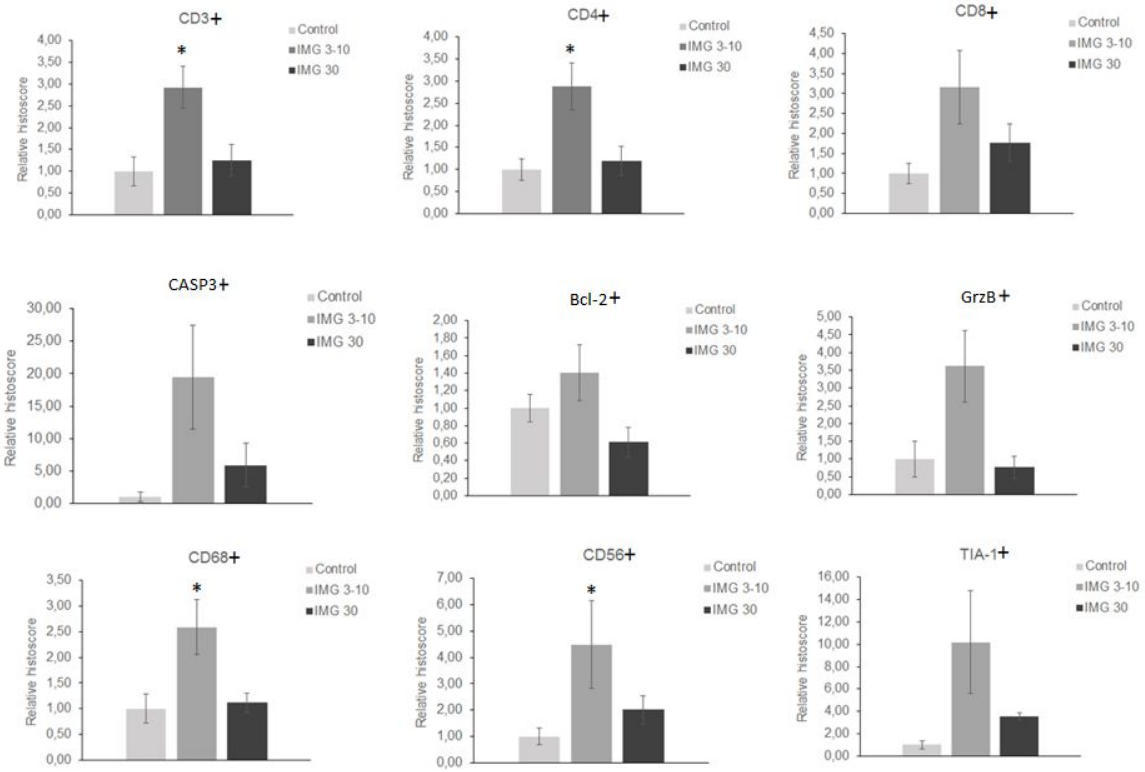

Figure 5. Bar chart with the results of all analyzed markers. ANOVA statistical test, * =P<0.05.

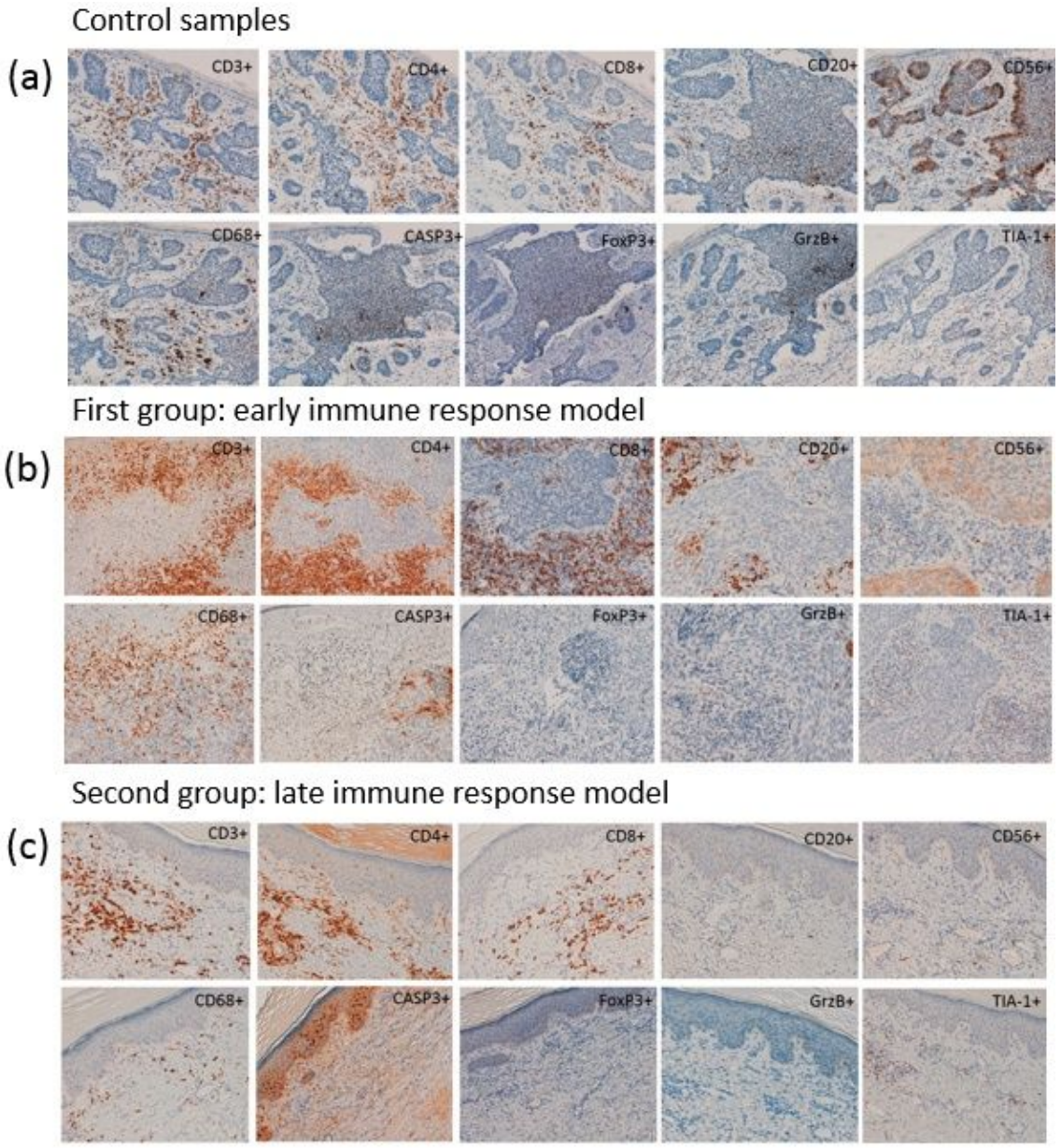

Figure 6. Representative samples showing immunohistochemistry results in the control samples and in the different groups. (a) Control samples. (b) Group 1(early immune response). (c) Group 2 (late immune response).

Antigen	Clone	Catalog number	Dilution	Source	Cell marker
CD3	Polyclonal	IR503	Ready to use	DAKO, Glostrup, Denmark	T lymphocytes
CD4	4B12	IR649	Ready to use	DAKO, Glostrup, Denmark	Helper lymphocytes
CD8	C8/144B	IR623	Ready to use	DAKO, Glostrup, Denmark	Cytotoxic lymphocytes
CD20	L26	IR604	Ready to use	DAKO, Glostrup, Denmark	B lymphocytes
CD56	123C3	IR628	Ready to use	DAKO, Glostrup, Denmark	Natural killers lymphocytes
CD68	PG-M1	IR613	Ready to use	DAKO, Glostrup, Denmark	Macrophages
Bcl-2	124	IR614	Ready to use	DAKO, Glostrup, Denmark	Antiapoptotic protooncogen
Cleaved caspase 3 (CASP3)	Polyclonal	9661	1:100	Cell signaling, Massachusetts, USA	Apoptotic marker
FoxP3	D2W8E	98377	1:100	Cell signaling, Massachusetts, USA	Regulatory lymphocytes
Granzyme B (GrzB)	GrB-7	M7235	1:50	DAKO, Glostrup, Denmark	Cytotoxicity marker
TIA1	TIA-1	Ab2712	1:100	Abcam, Cambridge, UK	Natural killers and cytotoxic lymphocytes

Table 1. Primary antibodies used in the study to evaluate inflammatory cell infiltration in groups 1 and 2 after treatment with IMG.

Patients	n (%)
Men	6 (37.5%)
Women	10 (62.5%)
Mean Age	66
BCC subtype	n (%)
Superficial	10 (62.5%)
Nodular	5 (31.25%)
Infiltrative	1 (6.25%)
Localization	n (%)
Trunk	9 (56.25%)
Abdomen	1 (6.25%)
Neck	2 (12.5%)
Upper extremities	0
Lower extremities	4 (25%)

Table 2. Clinical and pathological data from the 16 patients with BCC included in the study.